Automated Protein Expression and Purification of NEBExpress® Cell-free *E. coli* Protein Synthesis Reactions using NEBExpress Ni-NTA Magnetic Beads

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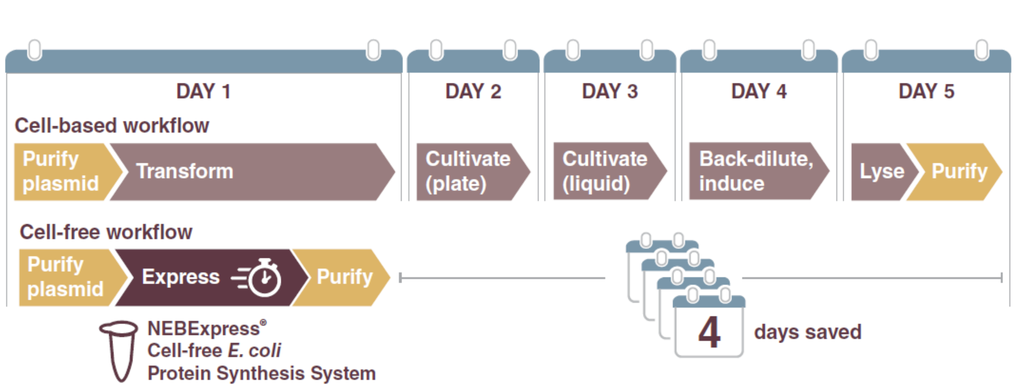
Introduction & Workflow

The unparalleled access modern researchers have to efficient and cost-effective DNA assembly methods (e.g. NEBuilder® Hifi DNA Assembly, NEBridge® Golden Gate Assembly) has rendered traditional protein expression and purification the main hurdles to high-throughput screening efforts. Transforming recombinant vectors into specific expression strains and cultivating precise biomass to reproducibly induce expression is too inexact and difficult to automate. Furthermore, traditional column purification is inherently single-stream, thus lacking high-throughput compatibility and limiting project scope. To overcome these issues, researchers have turned to cell-free expression methods and affinity purification with functionalized magnetic beads to streamline and automate high-throughput efforts to rapidly generate analytical amounts (~0.5 mg/mL) of diverse target proteins in hours instead of days (Figure 1).

FIGURE 1: **Timeline of traditional expression and purification vs. cell-free protein synthesis and automated Ni-NTA magnetic bead purification**

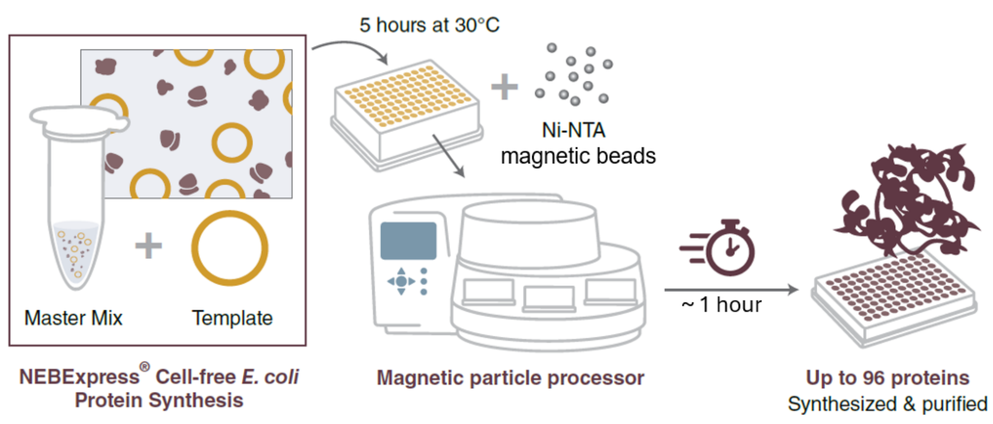


Cell-based workflow: A recombinant plasmid that directs expression is purified and transformed into an appropriate host strain and plated overnight. Colonies are picked the next day and grown overnight in liquid media. The overnight culture is back-diluted, grown to mid-log phase (OD = 0.4-0.8) and induced. The culture is pelleted the next day, lysed, and purified via column chromatography.

Cell-free workflow: A recombinant plasmid that directs expression is purified and mixed with NEBExpress Cell-free *E. coli* Protein Synthesis master mix, incubated for a few hours, mixed with NEBExpress Ni-NTA Magnetic Beads, and loaded onto a magnetic particle processor for automated purification.

NEB offers agarose-based, super-paramagnetic Ni-NTA beads, known as NEBExpress Ni-NTA Magnetic Beads (NEB #S1423S/L), for small-scale purification of polyhistidine-tagged (His-tagged) proteins. Since these beads can be easily dispensed to 96 well plates, they are particularly amenable to purifying synthesized His-tagged proteins from NEBExpress Cell-free *E. coli* Protein Synthesis reactions in a high-throughput manner. We aimed to take this one step further by automating the purification using a magnetic particle processor (MPP) as depicted in the workflow in Figure 2. The MPP automates the purification procedure by transferring the magnetic beads between plates containing purification buffers. In this case, his-tagged proteins bind to the magnetic beads, the MPP transfers the beads to plates containing wash buffer and then to a plate containing elution buffer to elute up to 96 purified proteins. **Herein, we detail a workflow incorporating a magnetic particle processor, NEBExpress Ni-NTA magnetic beads, and NEBExpress Cell-free *E. coli* Protein Synthesis reactions that enables users to go from DNA to *purified* proteins in a single day with less hands-on time and greater reproducibility.**

FIGURE 2: **Semi-automated workflow incorporating Cell-free Protein Synthesis, magnetic beads and a magnetic particle processor**



NEBExpress Cell-free *E. coli* Protein Synthesis Mastermix is assembled and dispensed to a 2 mL 96-well plate. Template encoding his-tagged protein is added to each well, and the reactions are incubated for 5 hours at 30C with agitation. NEBExpress Ni-NTA magnetic beads are then added to each well, and the plate is placed in the magnetic particle processor, which performs the binding, wash, and elution steps in approximately 1 hour, yielding up to 96 purified proteins.

Methods

Cell-free protein synthesis reaction assembly, dispensing, and SDS-PAGE analysis

NEBExpress Cell-free *E. coli* Protein Synthesis System reactions were assembled (Table 1) for seven His-tagged target proteins and a no template control (Table 2) in 100 µL total volume, which is twice the volume of a typical reaction. This reaction volume was used based on our established protocols; however, the reaction volume is scalable from 10 uL up to the maximum recommended volume of the specific magnetic particle processor and plates used. Six replicates of each sample were included to determine reproducibility, resulting in 48 total reactions. A master mix of NEBExpress Cell-free *E. coli* Protein Synthesis System components excluding template DNA was assembled for 55 reactions (48 rxns + 15% overage) in a 5 mL tube. The master mix was bulk dispensed (90 µL/well), followed by addition of 500 ng/well plasmid DNA or H_2O that was multi-dispensed using an 8-channel repeat pipettor. The deep-well plate was sealed and incubated in a ThermoMixer™ C for 5 hours at 30°C with 500 rpm shaking. Prior to purification, representative samples were retrieved and run on a 10-20% Tris-Glycine SDS-PAGE gel, and gel images were captured with a LI-COR Odyssey® M imager to visualize target synthesis prior to automated affinity purification (Figure 3A).

Automated protein purification using Ni-NTA magnetic beads and a magnetic particle processor

For purification of proteins from the NEBExpress Cell-free *E. coli* Protein Synthesis reactions, NEBExpress Ni-NTA Magnetic Beads were equilibrated with and then resuspended in 2X IMAC buffer. The equilibrated beads were diluted with 1X IMAC containing 40 mM imidazole, bulk dispensed with a tip-based liquid handler to each sample well of the reaction plate, and the target proteins were purified using the Thermo Scientific™ KingFisher™ Flex System. For the KingFisher™ procedure, equilibrated beads were mixed with the NEBExpress Cell-free *E. coli* Protein Synthesis System reactions for 30 min at 30°C (Plate #1), washed twice for 5 min in 500 µL/well 1X IMAC Buffer supplemented with 20 mM imidazole (Plates #2-3), and eluted for 10 min in 75 µL/well of 1X IMAC Buffer supplemented with 250 mM imidazole (Plate #4). All steps were performed at room temperature, employed slow, continuous agitation, and utilized deep-well plates apart from the elution step (Plate #4), which employed a KingFisher™ 96-well microplate. Purity and yield of the purified protein targets were determined by analyzing 5 µL representative samples by SDS-PAGE (Figure 3B). Sample concentration and purity were additionally quantified on the Revvity LabChip® GXII Touch using the high-sensitivity protocol.

TABLE 1: **NEBExpress Cell-free *E. coli* Protein Synthesis System Master Mix Assembly**

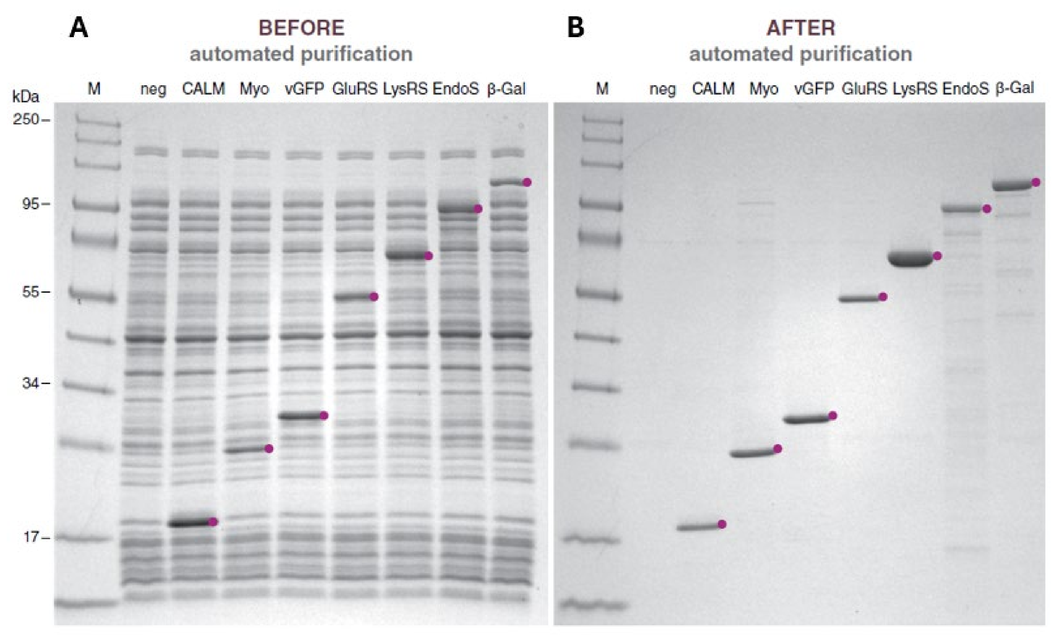
NEBEXPRESS CELL-FREE *E.COLI* PROTEIN SYNTHESIS REACTION COMPONENTS	STANDARD RXN (µl)	2X RXN (µl)	55X MASTER MIX (µl)	VOLUME PROVIDED IN E5360L KIT (µl)
Template (100 ng/µl)	5	10		
Protein Synthesis Buffer (2X) (#B0864SVIAL)	25	50	2,750	2,500
NEBExpress S30 Synthesis Extract (#P0864SVIAL)	12	24	1,320	1,250
RNase inhibitor, Murine (#M1018AAVIAL)	1	2	110	150
T7 RNA polymerase (#M1019AAVIAL)	1	2	110	150
RNase-free water	6	12	660	
Total Volume (µl):	50	100	4,950	

TABLE 2: **His-tagged target proteins**

SAMPLE	PROTEIN	MOLECULAR WEIGHT (kDa)
1	–ctrl	–
2	CALM	16.7
3	Myokinase	25.5
4	vGFP	26.9
5	GluRS	54.9
6	LysRS	75.0
7	EndoS	89.0
8	β-Gal	100

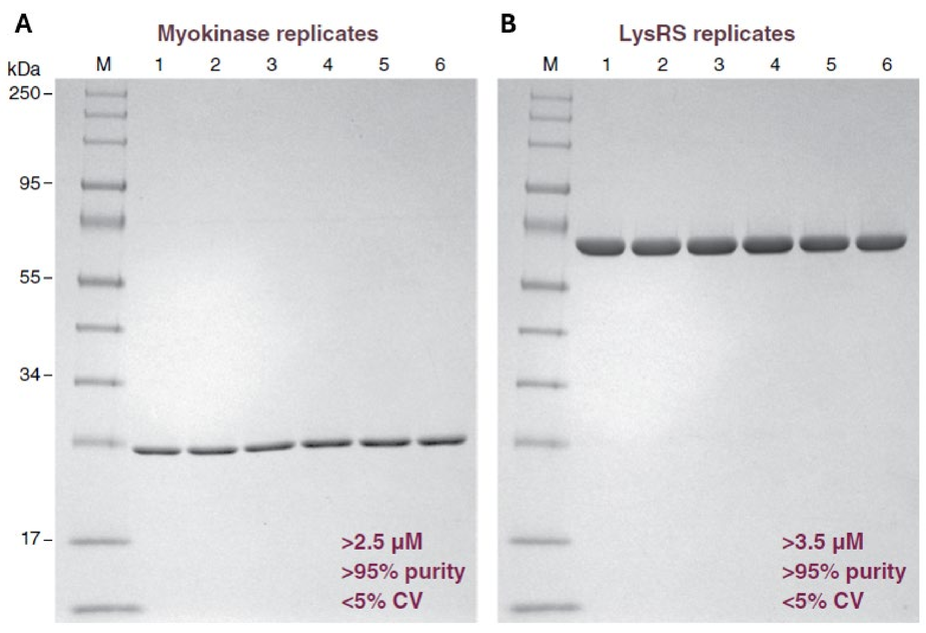
Results & Conclusions

FIGURE 3: **Proteins synthesized in NEBExpress Cell-free *E. coli* Protein Synthesis reactions and subsequently purified using NEBExpress Ni-NTA Magnetic Beads and a magnetic particle processor**



A) Synthesized proteins in NEBExpress Cell-free *E. coli* Protein Synthesis reactions before automated purification. B) Synthesized proteins in NEBExpress Cell-free *E. coli* Protein Synthesis reactions after automated purification. The red circle indicates the specific protein of interest. M = Color Prestained Protein Standard, Broad Range (NEB# P7719S/L). Representative synthesis and purified samples were run for the following templates: no template (neg), CALM (16.7 kDa) , Myokinase (25.5 kDa), vGFP (26.9 kDa), GluRS (54.9 kDa), LysRS (75.0 kDa), EndoS (89.0 kDa) and β-Gal (100.0 kDa).

FIGURE 4: **Experimental replicates of automated Ni-NTA magnetic-bead-purified NEBExpress Cell-free *E. coli* Protein Synthesis reactions**



Six independently assembled and purified replicates for A) Myokinase (25.5 kDa) and B) LysRS (75.0 kDa).

- **NEBExpress Cell-free *E. coli* Protein Synthesis System can be dispensed using standard tip-based liquid handlers into 96-well plates and perform robust protein synthesis (Figure 3A)**
- **Overall hands-on time of assembly and dispensing of approximately 10 minutes**
- **Go from template addition to purified protein in approximately 6 hours**
- **All seven purified target proteins displayed remarkable improvements in purity relative to the crude reactions (Figure 3B)**
- **High-quality protein of nearly identical yield and purity was purified for all six replicates (Figure 4).**
- **Average purity of >90% was achieved for all templates as determined by LabChip**

Automated purification of proteins synthesized in NEBExpress Cell-free *E. coli* Protein Synthesis reactions has the potential to revolutionize research by enabling rapid, reproducible, and high-throughput production of highly pure and diverse proteins. The NEBExpress Cell-free *E. coli* Protein Synthesis System is well-suited for protein screening since it offers high expression levels across a wide range of protein molecular weights, is scalable, tunable, reproducible, compatible with all common purification tags, able to synthesize proteins that exhibit toxicity *in vivo*, and cost-effective.

References

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4. **Magnelli, P. et al. (2020) Technical Note: *NEBExpress® Cell-free E. coli protein synthesis system—a high performance E. coli cell lysate-based system for in vitro protein synthesis.*** New England Biolabs, Inc.
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